Sulphate-activated phosphorylase *b*: the pH-dependence of catalytic activity

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The pH-dependence of sulphate-activated phosphorylase *b* has been studied in the direction of glycogen synthesis. The bell-shaped curve of the pH-dependence of the catalytic constant for the AMP-activated enzyme showed p*K* values of 6.1 and 7.3, but the curve for the enzyme activated by 0.9 M ammonium sulphate showed a drop of activity on the acid side at much higher pH values. Its bell was centred at pH 7.8 but it was too narrow to be characterized by only two p*K* values. The narrowness of the curve could be explained by positive co-operativity, but not its unusually steep acid side. We suggest that the fall on the acid side is due to more than one hydronation (addition of H⁺). The points can be fitted by a curve with two de-activating hydronations and a de-activating dehydronation having identical titration p*K* values of 7.5, and hence molecular values of 7.0, 7.5 and 8.0. If both 0.9 M ammonium sulphate and 5 mM AMP are added, the bell is as broad as with AMP alone, but is somewhat raised in pH optimum. The results are discussed in the light of new structural data from crystallographic studies on binary complexes of the enzyme.

INTRODUCTION

Muscle glycogen phosphorylase (EC 2.4.1.1) is a complex allosteric enzyme. It catalyses the phosphorolysis of glycogen in muscle and liver to yield glucose 1-phosphate. Its phosphorylated and unphosphorylated forms are known as phosphorylases *a* and *b* respectively. The enzyme is active as a dimer ($M_r = 195000$) but may also form tetramers that still retain some activity [1,2]. The structure of the R-state tetrameric phosphorylase *b*, based upon the X-ray analysis of crystals of the enzyme grown from ammonium sulphate solution, has been solved at 2.8 Å (1 Å = 10^{-10} m) [3,4]. The X-ray analysis revealed tertiary structural changes in response to sulphate binding. These changes, concomitant with conversion of the enzyme into the R-state, are coupled to large changes in quaternary structure which directly affect the AMP and the Ser-14 phosphate site and indirectly the catalytic site. Sulphate mimics phosphate by binding to the catalytic site, the AMP allosteric phosphate-recognition site and the Ser-14(*P*) site [3]. A detailed analysis of the effect of ammonium sulphate on the solution properties of phosphorylase *b* showed that ammonium sulphate can be considered as an allosteric effector since it activates the enzyme through stabilization of its active state [5]. The X-ray crystallographic studies have established that, associated with the binding of sulphate at the Ser-14(*P*) site, the N-terminal residues 10–18, which are poorly ordered and adopt an irregular extended conformation in the T-state phosphorylase *b*, become ordered. The conformational changes that occur at the sulphate-binding sites and in the N-terminal region, and in addition the change in the quaternary conformation, appear to be inter-related, in that there is a concerted tertiary and quaternary structural change resulting in an active conformation of the catalytic site and access for substrate [3,6–8]. It seems likely that the N-terminal residues play an important role in stabilizing the R-state; without them, sulphate alone may not be able to promote the R-state. Kinetic experiments performed on phosphorylase *b'*, a species (produced by proteolysis) that lacks the N-terminal 16 residues [9] and is inactive in the absence of nucleotides [10], showed that this form of the enzyme is no longer activated by ammonium sulphate [11]. Leonidas et al. [11] interpreted this lack of activation as indicating that sulphate activation of phosphorylase *b* occurs through binding to the Ser-14(*P*) site with concomitant and essential ordering of the N-terminus, and not as a result of binding to the allosteric and catalytic phosphate-recognition sites.

Studies of pH-dependence of enzyme-catalysed reactions can provide important information about the type of amino-acid side-chain groups that participate in catalysis [12–17]. The pH-dependence of enzyme activity often follows a bell-shaped curve. This is generally taken to imply that at least two ionizing groups are involved in catalysis, catalytic activity requiring one group to be hydronated and another to be dehydronated. If the pH-dependence of any physical quantity in a polyelectrolyte gives a simple curve like that of a monobasic acid, the p*K* found will be a 'titration' value, to which several groups may contribute (see Dixon [16], who also explains the relation between molecular and titration p*K* values). Hence attributing the p*K* to a single group may be wrong unless this group is distant enough from other ionizing groups of similar strength for its group p*K* not to be appreciably influenced by their state of ionization. The phosphorylase kinetic mechanism is rapid-equilibrium random Bi Bi [18,19]. Either glycogen or glucose 1-phosphate can bind first to the phosphorylase, and the resulting ternary complex of enzyme, glycogen and glucose 1-phosphate is then transformed into the ternary complex of enzyme, glycogen (with one more glucose unit) and phosphate. Several studies have been performed on the pH-dependence of the glycogen phosphorylase-catalysed reaction. The activity/pH profiles of all phosphorylases so far studied gave bell-shaped curves with similar molecular p*K* values [20–25]. Kasvinsky and Meyer [23] studied the pH–rate profile for glycogen phosphorylase from rabbit muscle in the direction of

glycogen degradation. They reported that the pH-dependence of the Michaelis constant for phosphate showed a pK of 6.56, which they interpreted as indicating that the di-anion was bound; they also found a bell-shaped curve for the limiting velocity, V, against pH, characterized by pK values of 6.1(2) and 7.0(3) at 30 °C, which could be ascribed to the enzyme–substrate complex. In the direction of glycogen synthesis, pK values between 5.7 and 6.4 for the acid and between 7.0 and 7.2 for the alkaline limb can be obtained from the results of Uhing et al. [24]. Similarly, in the work of Withers et al. [25], the pK values were about 5.3 and 7.4 respectively, as calculated by Fukui et al. [26]. These authors thought it likely that the pK (6.2) of the phosphate group of the pyridoxal phosphate was responsible for the acid limb, and that of a histidine residue for the alkaline limb. Tagaya and Fukui [27] found that the log k/pH plot for glucosyl transfer from phosphorylase b reconstituted with pyridoxal 5′-diphospho(1)-D-glucose gave pK values of 6.90 and 8.84 at 25 °C. These were ascribed to side-chains of residues, since in this derivative each phospho group (-O-PO$_2$$^-$-O-) can only be mono-anionic. They suggested that, in the normal reaction, the imidazolyl group of His-377 acts as a nucleophile attacking the anomeric carbon of the substrate glucose 1-phosphate. However, the strongest argument against a glucosyl histidine intermediate in phosphorylase is the finding that 1-α- and 1-β-D-glucopyranosyl-imidazoles are very stable species [28]. In addition, as indicated by X-ray crystallographic studies on the binary complexes of the enzyme, it is unlikely that His-377 is directly involved in the catalytic mechanism, although it is involved in substrate binding, by forming a hydrogen bond to O-6 of the sugar, through its imidazole nitrogen N$^{\delta 1}$, i.e. N$^{\pi}$ [6,29,30].

In this paper, the pH-dependence of the activity of phosphorylase b has been studied in order to investigate further the mechanism by which the enzyme is activated by ammonium sulphate. The kinetic results show that the catalytic activity of sulphate-activated phosphorylase b increases sharply with increasing pH in the range 6.4–7.8.

EXPERIMENTAL

Materials

Oyster glycogen (Sigma Chemical Co.) was freed of AMP by the method of Helmreich and Cori [31]. AMP, glucose 1-phosphate (dipotassium salt) and ammonium sulphate were products of Sigma Chemical Co., and glycerol 2-phosphate (disodium salt) was obtained from Merck.

Preparation of phosphorylase b

Phosphorylase b was isolated from rabbit skeletal muscle using the method of Fischer and Krebs [32] (using 2-mercaptoethanol instead of L-cysteine) and recrystallized at least four times. Bound nucleotides were removed from the enzyme as described by Melpidou and Oikonomakos [33].

Protein concentration

Protein was determined from absorbance measurements at 280 nm using an absorbance index $A^{1\%}_{1cm} = 13.2$ [34].

Determination of phosphorylase activity

Phosphorylase activity was measured at 30 °C in the direction of glycogen synthesis by the release of orthophosphate from glucose 1-phosphate. The buffers of the indicated pH contained 0.9 M ammonium sulphate (when added), 20 mM glycerol 2-phosphate,
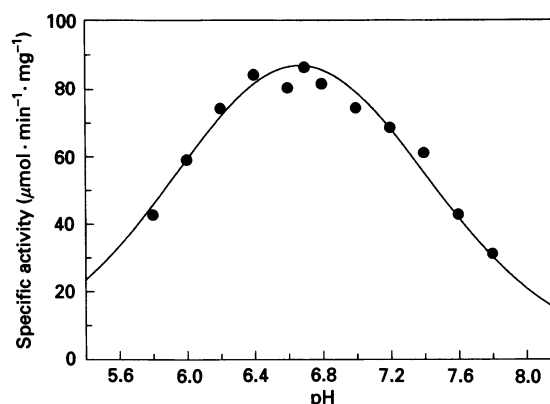

Figure 1   The pH-dependence of the specific activity of AMP-activated phosphorylase b

The enzyme (10 μg/ml) was assayed at constant concentrations of glucose 1-phosphate (60 mM), glycogen (1%) and AMP (5 mM). At these substrate concentrations, the rates of reaction are likely to approach their limiting values. The curve was calculated from the equation developed by Alberty and Massey [37] with pK values of 6.05 ± 0.06 and 7.29 ± 0.06, apparent maximal specific activity = 87.0 ± 1.5 μmol · mg^{-1} · min^{-1} or maximal specific activity = 128.7 ± 7.5 μmol · mg^{-1} · min^{-1}.

0.5 mM EDTA and 1 mM dithiothreitol. The purpose of using glycerol 2-phosphate was to keep conditions similar to those used previously either for ammonium sulphate activation of phosphorylase b [5] or for crystallographic experiments [3]. Substrate mixtures and buffers were adjusted to the indicated pH. The enzyme (10–100 μg/ml) was preincubated with glycogen for 15 min at 30 °C before the reaction was started by adding the glucose 1-phosphate. Under these assay conditions, the enzyme exists as a dimer [2]. Samples were withdrawn at 1 min intervals over the period from 1–5 min and transferred into 0.2% SDS to stop the reaction. Inorganic phosphate released in the phosphorylase reaction was determined by the method of Fiske and Subbarow [35].

Kinetic analyses

Initial rates of reaction, v, were calculated from the pseudo-first-order reaction constants [2,19] by using a non-linear-regression data analysis program (GraFit) and assuming that the standard error, σ^2, is the same for each data point ('simple weighting') [36]. The program calculated the reaction rates and the standard errors of these values. V (limiting rate of reaction) at each pH was obtained by applying the Michaelis–Menten equation to the kinetic data; initial rates were treated by non-linear explicit-weighting regression analysis [36], i.e. by providing an explicit value for the standard deviation of each rate. When v or V was plotted as a function of pH, the curves were calculated by applying either the equation derived by Alberty and Massey [37] or that derived by Dixon [38,39] added to GraFit using the built-in equation editor.

RESULTS

The enzymic activity of phosphorylase b in the presence of 5 mM AMP at various pH values was assayed. The curve of specific activity against pH fitted a curve (Figure 1) characterized by molecular pK values of 6.0 and 7.3, using the analysis of Alberty and Massey [37]; since the concentration of glucose 1-phosphate was 60 mM, and likely to be saturating, we interpret these as
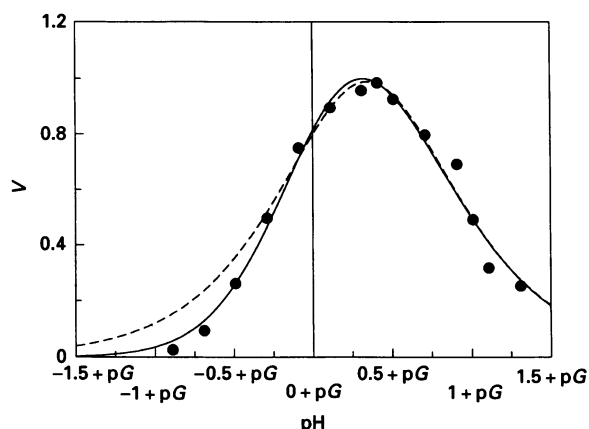
Figure 2   The pH-dependence of V of sulphate-activated phosphorylase b

The best three-proton fit (shown as a continuous line) was derived as described in the text by applying the equation derived by Dixon et al. [40] with molecular pK values of 7.0, 7.5 and 8.0. The curve was multiplied by $(m+n)^{(m+n)}/(m^m \cdot n^n)$ to normalize it to give a value of 1. The value of 1 corresponds to an apparent V_{max} value of 57.3 μmol $\cdot$ mg^{-1} $\cdot$ min^{-1}. The apparent value of V_{max} is the value that the active ionic form of the enzyme would have. The normalized value of V_{max} is 3.3, which corresponds to 190 μmol $\cdot$ mg^{-1} $\cdot$ min^{-1}. The best two-proton fit (shown as a broken line) was derived according to Alberty and Massey [37] with pK values of 7.9 and 7.8, apparent V_{max} = 56.7 μmol $\cdot$ mg^{-1} $\cdot$ min^{-1} and V_{max} = 193 μmol $\cdot$ mg^{-1} $\cdot$ min^{-1}. The enzyme (40 μg/ml) was assayed at 40–140 mM glucose 1-phosphate and a constant concentration of glycogen (1%) as described in the Experimental section. The ammonium sulphate concentration was 0.9 M.

values for the enzyme–substrate complex. Our pH–activity profiles for the enzyme in the presence of AMP agree with those previously reported [23–25].

The pH-dependence of the catalytic activity of the phosphorylase-catalysed reaction has been examined in the presence of 0.9 M ammonium sulphate (Figure 2). Here, V, obtained by extrapolating to saturating concentrations of glucose 1-phosphate, is plotted against pH. The pH profile of the sulphate-activated phosphorylase b differs from that of the AMP-activated phosphorylase b (Figure 1), by having a much higher pH optimum and by having very little activity at pH 6.4–6.8 where the AMP-activated form is most active. Since the fall of activity in the acid limb could result from inactivation of phosphorylase, the enzyme was preincubated at pH values between 6 and 7 and then assayed at pH 7.8. As full activity was regained, the fall on the acid side must result from reversible changes. The data for the sulphate-activated enzyme could not be fitted satisfactorily to a curve characterized by two pK values [37], in that activity fell off with acidification to a greater extent than the equation predicted (Figure 2). The sharpness of the peak could be explained if the two hydronations were positively co-operative, but this would not explain its skewness. If, however, three hydronations were involved, with two deactivating on the acid side of the optimum pH of 7.8, the steepness on this side might be explained. The curve was therefore calculated from the equation that represents the sharpest bell that can be obtained without postulating positive co-operativity of binding. This condition is met by letting all the hydronations have the same titration dissociation constant, G. Then the fractional concentration of the form H$_n$A^{m-} (which can be hydronated m times and dehydronated n times) is (see Dixon et al. [40], appendix 1) $[H^+]^n \cdot G^m/([H^+]+G)^{(m+n)}$. The velocity should be proportional to this. A satisfactory fit was obtained (Figure 2) with $m = 2.19$ and $n = 1.05$, suggesting that two hydronations deactivate on the acid side and one dehydronation
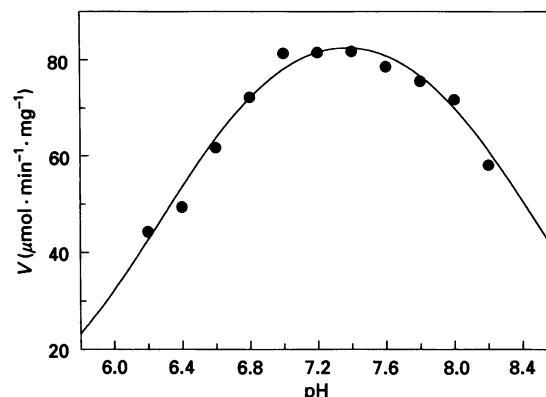

Figure 3   The pH-dependence of V of sulphate-activated phosphorylase b in the presence of 5 mM AMP

Glucose 1-phosphate concentration was 6–60 mM at constant concentrations of glycogen (1%) and ammonium sulphate (0.9 M). Enzyme concentration was 10 μg/ml throughout. The curve was calculated according to the equation of Alberty and Massey [37] with pK values of 6.32 $\pm$ 0.02 and 8.45 $\pm$ 0.06, apparent V_{max} = 82.9 $\pm$ 1.0 μmol $\cdot$ mg^{-1} $\cdot$ min^{-1} and V_{max} = 96.4 $\pm$ 1.2 μmol $\cdot$ mg^{-1} $\cdot$ min^{-1}.

deactivates on the alkaline side. The optimal pH is pG + log m − log n from which a pG value of 7.5 can be calculated, giving molecular values of 7.0, 7.5 and 8.0. The results obtained here show that ammonium sulphate activation of phosphorylase b raises the pK values markedly.

Figure 3 shows a V versus pH curve for sulphate-activated phosphorylase b in the presence of AMP. The pH optimum is at pH 7.4, which is rather similar to that of the sulphate-activated enzyme in the absence of AMP (Figure 2). The only notable difference between the two profiles is that the pK in the acid limb is shifted down to a value of 6.3, which is similar to that for the AMP-activated phosphorylase b, while the pK value in the alkaline limb is scarcely changed.

The pH profile of sulphate-activated phosphorylase b has been shifted markedly (with respect to the AMP-activated enzyme) to the alkaline side. This could be due either to the increased ionic strength resulting from the addition of ammonium sulphate, or to the conformational change induced by sulphate binding. This rise in pK values, at least for those on the acid side of the bell-shaped curve, is unlikely to be due to the raised salt concentration, since there is no similar rise on addition of sulphate to AMP-activated phosphorylase b.

DISCUSSION

The catalytic mechanism of phosphorylase has been extensively investigated and there are two main proposals for the role of the 5′-phosphate group of the coenzyme based on solution and crystallographic studies [7,41–44]: upon formation of the ternary complex it functions either as a proton donor–acceptor shuttle in general acid–base catalysis or as an electrophilic 'constrained' trigonal–bipyramidal di-anion to promote attack by the substrate phosphate (which is bound as the apical ligand) on the polysaccharide substrate. To illustrate this we looked at both mechanisms in the reverse direction, in which the substrate is glucose 1-phosphate. In the former case, the 5′-phospho group assists by supplying a hydron to receive some of the negative charge of the
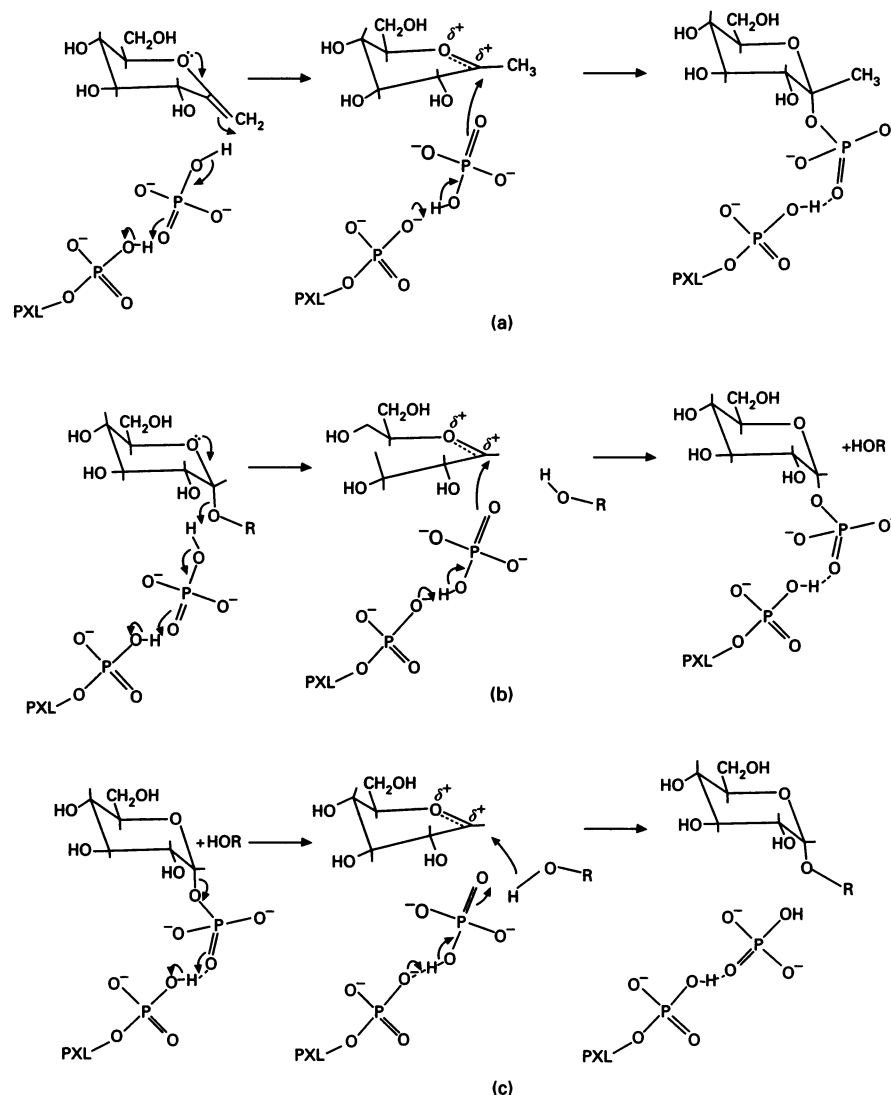
Figure 4   Proposed mechanism of phosphorylase catalysis

(a) Phosphorolysis of heptenitol; (b) phosphorolysis of oligosaccharide; (c) oligosaccharide synthesis [45]. PXL, pyridoxal.

phospho group as it leaves glucose 1-phosphate; in the latter case, this group assists by receiving some of this negative charge from an oxygen of the phospho group that is being transferred, and it does so at its central phosphorus atom, which bears a partial positive charge. Both proposals depend on the correct positioning of the substrate phosphate with respect to the cofactor 5'-phosphate. The strongest evidence for the direct phosphate–phosphate interaction between cofactor and product phosphate comes from the crystallographic analysis of the T-state complex between phosphorylase b and heptulose 2-phosphate [45]. The structural results showed no group (other than the cofactor 5'-phosphate) that could act as hydron donor or electrostatic stabilizing group in the vicinity of the catalytic site. The close proximity of the product phosphate to the cofactor 5'-phosphate provides support for a mechanism in which phosphorolysis of heptenitol is catalysed by general acid attack of the substrate phosphate, promoted by the cofactor 5'-phosphate (Figure 4a). This proposal is also supported by studies with R-state phosphorylase b. In the R-state crystals of tetrameric phosphorylase

b, obtained in the presence of 1.0 M ammonium sulphate [3], there is a sulphate bound at the catalytic site whose position is suitable for the attacking phosphate in the enzyme-catalysed phosphorolysis of heptenitol. In the sulphate-activated R-state complex between phosphorylase b and glucose 1-phosphate (Figure 5), the interactions between the glucosyl portion and the enzyme are similar to those of heptulose 2-phosphate [45] and glucose 1-phosphate [29] with the T-state enzyme. There is no direct contact between the substrate phosphate and the cofactor 5'-phosphate group; the observed distance between the phosphorus atoms is 6.4 Å. This value is similar to that observed in the complex with glucose 1-phosphate (6.1 Å) and larger than that in the complex with heptulose 2-phosphate (4.8 Å) in the T-state. There are small conformational changes between the R-state complex with glucose 1-phosphate and the native R-state that include a shift of Arg-569 (1.8 Å), shifts in Lys-568 so as to weaken the contact between Lys-568 and the cofactor 5'-phosphate and to make a hydrogen-bond with Glu-672, and a shift in Lys-574 away from Glu-672 and towards the cofactor 5'-
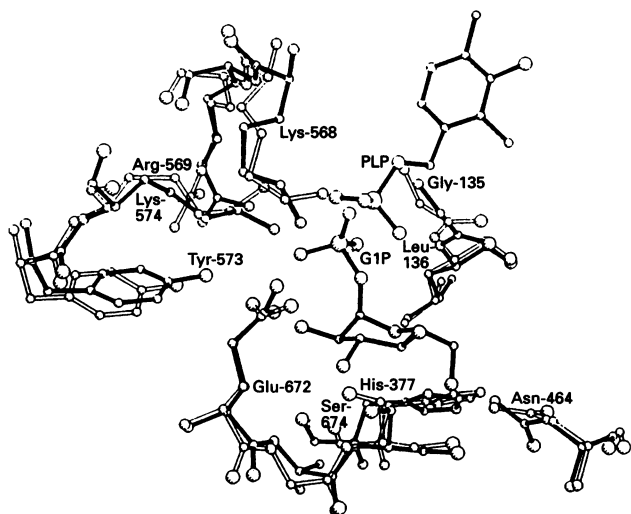
Figure 5   Comparison of the sulphate-activated R-state phosphorylase *b* (white structure) and the complex between phosphorylase *b* and glucose 1-phosphate (black structure)

The substrate phosphate makes polar contacts to Arg-569 and to the main-chain N of Gly-135 and Leu-136 but not to Lys-574, which shifts towards the cofactor 5′-phosphate [6]. Pyridoxal 5′-phosphate (PLP) of the sulphate-activated R-state phosphorylase *b* has been omitted for clarity. The crystallographic experiment was done by soaking native phosphorylase *b* crystals in a buffered solution containing 1.0 M sodium tartrate (in order to displace the sulphate from the catalytic site), 50 mM ammonium sulphate [to keep the Ser-14(*P*) site saturated with sulphate], 10 mM glycerol 2-phosphate and 200 mM glucose 1-phosphate, pH 7.5 [6]. Under these conditions, the unit cell *c*-dimension doubled from 88.2 to 176.4 Å, giving rise to eight subunits per asymmetric unit. The crystal structure of the R-state glucose 1-phosphate complex was refined using X-PLOR energy and crystallographic least-squares minimization as described by Hu [6]. The crystallographic *R* factor for 143747 reflections ($F > \sigma_F$) at 2.9 Å resolution is 0.188. The model consists of 54522 non-hydrogen atoms including sulphate and glucose 1-phosphate (G1P) atoms and 640 water molecules. The root mean square (rms) deviations from ideal bond lengths and angles are 0.021 Å and 4.3 ° respectively. The estimated error in the coordinates is between 0.2 and 0.3 Å, and the positions of the Cα atoms for all eight subunits deviate from their mean positions by 0.35 Å (rms), a value that indicates that the subunits are identical in their overall conformation to within the limits of the 2.9 Å resolution data [6].

phosphate [6]. The reaction mechanism postulated for phosphorolysis of heptenitol (Figure 4a) can be extended to the reaction with the natural substrates (Figures 4b and 4c). In the natural reaction with an oligosaccharide substrate it seems likely that the presence of the oligosaccharide in the catalytic site would favour a conformation in which the substrate phosphate is directed towards that observed in the complex with heptulose 2-phosphate so that the cofactor 5′-phosphate can function as a catalytic group [45]. It is therefore reasonably certain that the relative positions of the 5′-phosphate group and the substrate phosphate are crucial in the catalytic mechanism of this enzyme with natural substrates. However, the mechanistic details that account for the allostery and pH-dependence are by no means clear.

Thus, despite the fact that so much evidence points towards the involvement of the 5′-phosphate group in catalysis, no satisfactory explanation has been given for the p*K* values observed with AMP-activated phosphorylase *b*. We suggest that, in the direction of glycogen synthesis, the p*K* values of 7.0–7.4 observed by Uhing et al. [24], Withers et al. [25] and in this study, could be assigned to the cofactor 5′-phosphate, while the p*K* value of approximately 6.0 could be assigned either to the substrate glucose 1-phosphate or to the cluster of interacting groups. Our kinetic experiments indicate that the presence of

ammonium sulphate increases the p*K* value(s) that characterize the acid limb of the pH profile. A good fit is obtained by postulating that two hydronations are involved. Without ammonium sulphate, one has a molecular p*K* of 6.0 and the other must be below this, since it is not apparent in the pH profile; addition of ammonium sulphate raises them to 7.0 and 7.5. These p*K* changes are one element in the complex process of activation of the enzyme by ammonium sulphate. We do not yet know how these changes in the ionization state of unidentified groups in the enzyme are connected with the molecular re-arrangements associated with the sulphate-induced activation of the enzyme. Slight changes in the interactions between glucose 1-phosphate and phosphorylase, in the presence of ammonium sulphate, such as those observed in the R-state phosphorylase *b*–glucose 1-phosphate complex (Figure 5), might result in a shift of the *V*/pH profile. However, the change from a two-proton to a three-proton mechanism could equally well suggest that the observed ionizations merely indicate changes between different conformers of this complex allosteric enzyme. Leonidas et al. [5] have proposed that the ammonium sulphate by itself does not trigger the true R-state conformation of the dimeric enzyme. So far there have been no crystallographic studies of the enzyme with an oligosaccharide bound at the catalytic site. It should also be emphasized that in our experiments the enzyme was in a dimeric state [2], since the activity of phosphorylase *b* was measured by pre-incubating the enzyme with glycogen before initiating the reaction with the substrate mixture. In the absence of structural data concerning the ternary complex of the dimeric enzyme with its natural substrates, a detailed description, in molecular terms, of the pH-dependence we have observed, cannot be given. We have seen that the addition of AMP to the sulphate-activated phosphorylase *b* appreciably lowers these p*K* values, so that they all fall to 6.3 or below. We have not been able to rationalize this result on the basis of current crystallographic knowledge. Crystallography of the complex formed between AMP and phosphorylase *b* (R-state) in ammonium sulphate [4] showed a few structural differences in the region of the allosteric site (around Tyr-75), but no detectable changes at the Ser-14(*P*) site or the catalytic site. The crystallographic studies were carried out at pH 7.5, corresponding to the alkaline limb of the activity profiles. It would therefore be interesting to have crystallographic data on the AMP–enzyme complex at lower pH, as structural changes may be observed that might be related to the p*K* shifts we report here. The fitting of the asymmetrical pH profile for the sulphate-activated enzyme has been achieved by postulating that two hydronations are responsible for the fall of activity on the acid side of the pH optimum. This treatment may be useful for other enzymes.

REFERENCES

1   Graves, D. J. and Wang, J. H. (1972) in The Enzymes (Boyer, P. D., ed.), 3rd edn., Vol. 7, pp. 435–482, Academic Press, New York
2   Leonidas, D. D., Oikonomakos, N. G., Papageorgiou, A. C. and Sotiroudis, T. G. (1992) Protein Sci. **1**, 1123–1132
3   Barford, D. and Johnson, L. N. (1989) Nature (London) **340**, 609–616
4   Barford, D., Hu, S.-H. and Johnson, L. N. (1991) J. Mol. Biol. **218**, 233–260
5   Leonidas, D. D., Oikonomakos, N. G., Papageorgiou, A. C., Xenakis, A., Cazianis, C. T. and Bem, F. (1990) FEBS Lett. **261**, 23–27
6   Hu, S.-H. (1991) Ph.D. Thesis, University of Oxford
7   Johnson, L. N. (1992) FASEB J. **6**, 2274–2282
8   Johnson, L. N. and Barford, D. (1993). Annu. Rev. Biophys. Biomol. Struct. **22**, 199–232

9   Fischer, E. H., Graves, D. J., Crittenden, E. R. S. and Krebs, E. G. (1959) J. Biol. Chem. **234**, 1698–1704

10 Janski, A. M. and Graves, D. J. (1979) Biochemistry **254**, 4033–4039

11 Leonidas, D. D., Oikonomakos, N. G. and Papageorgiou, A. C. (1991) Biochim. Biophys. Acta **1076**, 305–307

12 Dixon, M. and Webb, E. C. (1964) The Enzymes (Boyer, P. D., ed.), 2nd edn., pp. 116–145, Academic Press, New York

13 Knowles, J. R. (1976) CRC Crit. Rev. Biochem. **4**, 165–173.

14 Tipton, K. F. and Dixon, H. B. F. (1979) Methods Enzymol. **63**, 183–234

15 Cornish-Bowden, A. and Wharton, C. W. (1988) Enzyme Kinetics, pp. 47–58, IRL Press, Oxford, Washington, DC

16 Dixon, H. B. F. (1992) Essays Biochem. **27**, 161–176

17 Brocklehurst, K. (1994) Protein Eng. **7**, 291–299

18 Engers, H. D., Bridger, W. A. and Madsen, N. B. (1969) J. Biol. Chem. **244**, 5936–5942

19 Engers, H. D., Shechosky, S. and Madsen, N. B. (1970) Can. J. Biochem. **48**, 746–754

20 Helmreich, E. and Cori, C. F. (1964) Proc. Natl. Acad. Sci. U.S.A. **52**, 647–654

21 Holo, J., Laszlo, E. and Juhasy, J. (1967) Staerke **19**, 285–290

22 Chao, J. and Graves, D. J. (1970) Biochem. Biophys. Res. Commun. **40**, 1398–1403

23 Kasvinsky, P. J. and Meyer, W. L. (1977) Arch. Biochem. Biophys. **181**, 616–631

24 Uhing, R. J., Lentz, S. R. and Graves, D. J. (1981) Biochemistry **20**, 2537–2544

25 Withers, S. G., Shechosky, S. and Madsen, N. B. (1982) Biochem. Biophys. Res. Commun. **108**, 322–328

26 Fukui, T., Tagaya, M., Takagi, M. and Shimomura, S. (1984) in Chemical and Biological Aspects of Vitamin B_6 Catalysis, Part A (Evangelopoulos, A. E., ed.), pp. 161–170, Alan R. Liss, New York

27 Tagaya, M. and Fukui, T. (1984) J. Biol. Chem. **259**, 4860–4865

28 Bourne, E. J., Finch, P. and Nagpurkar, A. G. (1972). J. Chem. Soc. Perkin Trans. 1, 2202–2205

29 Martin, J. L., Johnson, L. N. and Withers, S. G. (1990) Biochemistry **29**, 10745–10757

30 Johnson, L. N., Hu, S.-H. and Barford, D. (1992) Faraday Discuss. **93**, 131–142

31 Helmreich, E. and Cori, C. F. (1964) Proc. Natl. Acad. Sci. U.S.A. **51**, 131–138

32 Fischer, E. H. and Krebs, E. G. (1962) Methods Enzymol. **5**, 369–373

33 Melpidou, A. E. and Oikonomakos, N. G. (1983) FEBS Lett. **154**, 105–110

34 Kastenschmidt, L. L., Kastenschmidt, J. and Helmreich, E. (1968) Biochemistry **7**, 3590–3608

35 Fiske, C. H. and Subbarow, Y. (1925) J. Biol. Chem. **66**, 375–400

36 Leatherbarrow, R. J. (1992) GraFit Version 3.0, Erithakus Software, Staines, U.K.

37 Alberty, R. A. and Massey, V. (1954) Biochim. Biophys. Acta **13**, 347–353

38 Dixon, H. B. F. (1979) Biochem. J. **177**, 249–250

39 Dixon, H. B. F. (1988) Biochem. J. **253**, 911–913

40 Dixon, H. B. F., Clarke, S. D., Smith, G. A. and Carne, T. K. (1991) Biochem. J. **278**, 279–284

41 Madsen, N. B. and Withers, S. G. (1986) in Coenzymes and Cofactors: Pyridoxal Phosphate and Derivatives (Dolphin, D., Poulson, R. and Avramovic, O., eds.), pp. 1–29, Wiley, New York

42 Johnson, L. N., Hajdu, J., Acharya, K. R. et al. (1989) in Allosteric Enzymes (Herve, G., ed.), pp. 81–127, CRC Press, Boca Raton, FL

43 Palm, D., Klein, H. W., Schinzel, R., Buehner, M. and Helmreich, E. J. M. (1990) Biochemistry **29**, 1099–1107

44 Oikonomakos, N. G., Acharya, K. R. and Johnson, L. N. (1992) in Post-translational Modification of Proteins (Harding, J. J. and Crabbe, M. J. C., eds.), pp. 81–151, CRC Press, Boca Raton, FL

45 Johnson, L. N., Acharya, K. R., Jordan, M. D. and McLaughlin, P. J. (1990) J. Mol. Biol. **211**, 645–661